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14. ABSTRACT

Over 200,000 new cases of invasive breast cancer are diagnosed in the United States each year and account for approximately 40,000 deaths. From a treatment perspective, breast cancer is a paradigm for individualized medicine with two personalized therapies in use; endocrine therapy for hormone receptor positive patients and HER2-targeted agents such as trastuzumab for HER2+ patients. However, patients with TNBC (basal-like subtype lacking all three receptors (ER, PR and Her2/neu) are refractory to these therapies. Surgical resection and standard chemotherapy regimens remain the only therapeutic options for women with TNBC, and these treatments usually fail resulting in an aggressive metastatic relapse and short overall survival. Therefore, there is an urgent need to develop new-targeted therapeutic approaches. **This proposal provides a mechanism-based approach, which promises to impact the treatment of TNBC, a subtype of highly metastatic breast cancer that confers the worst outcome.**

We have identified miR-708 as a potential “metastasis suppressor” in breast cancer. miR-708 targets neuronatin to decrease intracellular calcium level, which inactivates ERK/FAK pathways to impair cell migration and metastases. Analysis of miR-708 upstream regions showed enrichment of PRC2 which was associated with elevated H3-K27me3 levels. We hypothesize that PRC2-induced H3-K27me3 silences miR-708 in metastasis. Significantly, systemic delivery of synthetic miR-708 blocked TNBC metastases, providing a rationale for developing miR-708 as a novel therapeutic agent against metastatic breast cancer. Our objective is to dissect the epigenetic regulation of miR-708, so that epigenetic therapies can be considered for metastatic breast cancer, and evaluate the therapeutic efficacy of synthetic miR-708. Dissecting the epigenetic regulation of miR-708 will generate translational opportunities for patients with TNBC. For example, insights into the role of PRC2 to directly mediate miR-708 silencing will allow the evaluation of epigenetic therapy in metastatic breast cancer. Our demonstration that restoration of miR-708 attenuates metastasis following metastatic colonization suggests the possibility of directly using miR-708 as a therapeutic modality. Of note, treatment with miR-708 is likely to target the more aggressive metastatic breast cancer cells that lack miR-708 and not affect normal tissues that maintain expression of miR-708.

In summary, the therapeutic potential of miR-708 may lead to the design of future clinical trials for the treatment of extraordinarily high-risk breast cancer patients whose tumor has undergone metastatic dissemination (Stage 4, NED, TNBC). Given the strong preclinical data that would emerge from this grant, we expect that with a rapid clinical translation the approximate time for these potential therapeutics to move from bench to bedside will be about 5-10 years.

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1. INTRODUCTION:

This proposal aims to dissect the epigenetic regulation of miR-708 by EZH2, so that epigenetic therapies can be considered for metastatic breast cancer. In addition, it will also evaluate the therapeutic efficacy of synthetic miR-708 in the treatment of breast cancer metastasis. The research plan is based on the hypothesis that PRC2-induced H3-K27me3 silences miR-708 in metastasis. The project's specific aims are (1) to determine the mechanism by which PRC2 complex regulates miR-708 in breast cancer metastasis and (2) to assess the therapeutic potential of miR-708 against metastatic triple-negative (TN) breast cancer using nanoparticle delivery system. A major goal of this study is that it aims to provide a targeted therapy for TN breast cancer, which has no current targeted therapy.

2. KEYWORDS:

Breast cancer, Metastasis, miR-708, Epigenetic, Nanoparticle, Targeted therapy.

3. ACCOMPLISHMENTS:

SOW AIM 1:

PRC2 Knockdown and evaluate miR-708, Nnat, migration in vitro

Subaim 1.1: Perform shRNA-mediated suppression of Suz12, Ezh2 in breast cell lines (months1-6): Completed (see Fig. 1)

We had previously identified mR-708 in TNBC, and shown that miR-708 is silenced by the PRC2 complex (Ryu et al., 2013). In the grant application, using ChIP experiments, we had shown enrichment of PRC2 in regions upstream of miR-708. PRC2 enrichment was associated with increased H3-K27me3. Based on these observations, we had hypothesized that PRC2-induced H3K27me3 results in the suppression of miR-708 in metastasis. Here, we show that EZH2 levels are elevated in TNBC cells with high metastatic potential compared to non-metastatic counterparts, and miR-708 expression levels show an inverse correlation to EZH2 (**Fig. 1A**). Note that although MDA-361 (ER+PR-) was derived from brain metastases occurring in a breast cancer patient, they are the least aggressive (Zhang et al., 1991), and do not metastasize from orthotopic sites. shRNA-mediated knockdown of EZH2 resulted in cell migration/invasion defects (data not shown).

To demonstrate that the PRC2 is directly responsible for silencing miR-708, shRNA-mediated knockdown EZH2 (>2 shRNAs), showed that EZH2 *knockdown* restores miR-708 expression in MDA-MB-231-LM2 cells (**Fig. 1B**).

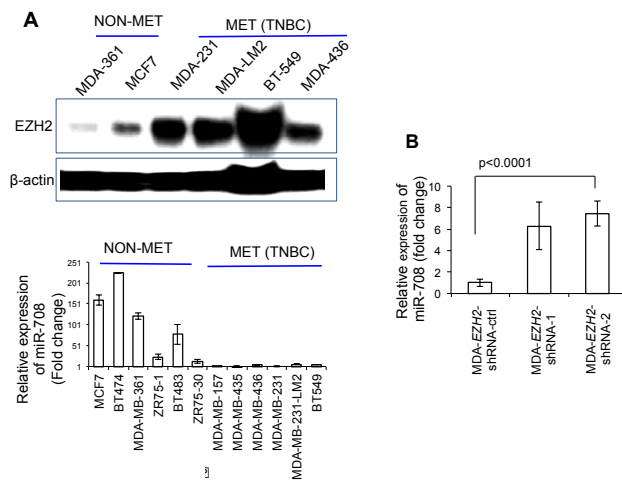


Fig. 1. EZH2-miR-708 axis in breast cancer metastasis. (A) EZH2 and miR-708 expression in TNBC breast cancer cell lines (upper and lower panels respectively), (B) Conditional knockdown of EZH2 with EZH2-shRNA restores miR-708 expression.

Subaim 1.2: Evaluate consequence on miR-708 and Nnat levels, cell migration (months 6-18) Completed (see Fig. 2 and Fig. 3)

We realize that shRNA-mediated knockdown of EZH2 destabilizes/degrades the PRC2 complex, and specific effects of EZH2 HMT on ensuing phenotypes cannot be deciphered. Therefore, to achieve specific and direct inhibition of the EZH2 activity, we used GSK126, a specific pharmacological inhibitor of EZH2 HMT (McCabe et al., 2012). Dose optimization studies showed that GSK at 2-5 μ M effectively inhibits EZH2 HMT in vitro (data not shown). Importantly, GSK126 specifically blocked EZH2-mediated H3K27 trimethylation (and not the related H3K4me trimethylation), and did not significantly affect EZH2 protein (Fig. 2A) indicating marked specificity. GSK126 suppressed migration (Fig. 2B-C), and invasion of metastatic breast cancer cells (Fig. 2E) without significant effects on proliferation (Fig. 2F). GSK126 did not impact primary breast tumor growth (data not shown).

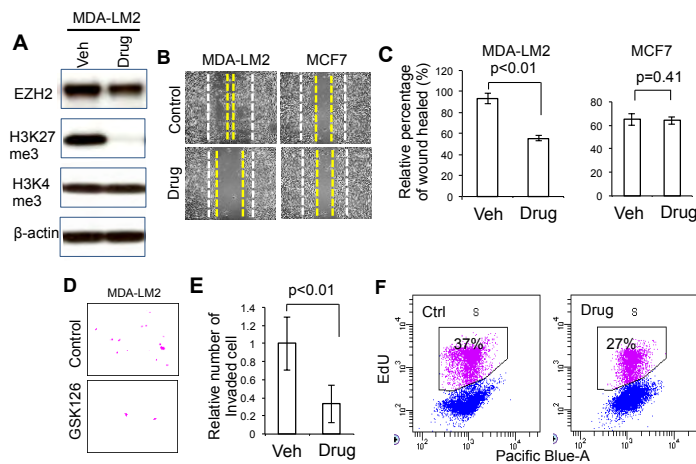


Fig. 2. EZH2 blockade impacts cell migration and invasion. (A) Metastatic breast cancer cells treated with vehicle (Veh) or EZH2 HMT inhibitor (GSK126, 5 μ M) for 3 days. Metastatic breast cancer cell MDA-LM2 or non-metastatic breast cancer MCF7 treated with vehicle (Veh) or EZH2 HMT inhibitor (Drug, 5 μ M) for 3 days. Effect of the drug on cell migration (A-B), invasion through a matrigel coated transwell (C-D), and proliferation.

Pharmacological inhibition of EZH2 restores miR-708 expression and inhibits Nnat: Consistent with

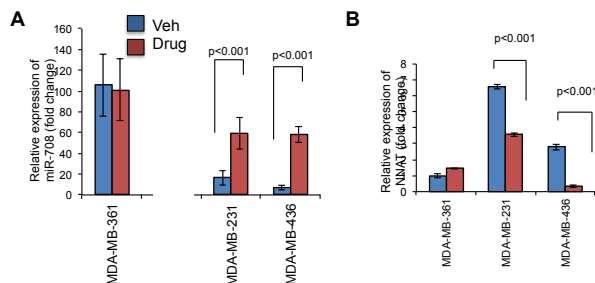


Fig. 3. EZH2 inhibitor restores miR-708 expression and inhibits Nnat. (A) 5 μ M GSK126 restores miR-708 expression in metastatic cell lines, MDA-MB-231 and MDA-MB-436 at day 10. Non-metastatic MDA-361 (Ezh2 low) expresses high levels of miR-708 and remains unaffected by GSK126. (C) Restoration of miR-708 results in concomitant downregulation of its target Nnat.

the genetic analysis that EZH2 knockdown restores miR-708 expression (Fig. 2A), pharmacological inhibition of EZH2 by administration of GSK126 restored miR-708 expression in metastatic TNBC cells and not in non-metastatic cells, which express high steady state levels of miR-708 (Fig. 3A). Importantly, restoration of miR-708 expression was associated with a decrease in the levels of Nnat (miR-708 target) (Fig. 3B).

Subaim 1.3: Evaluate consequence on metastasis in vivo (months 6-24): In Progress (see Fig. 4).

We have found that siRNA-mediated EZH2 knockdown restored expression of miR-708 (Fig. 1B), however these cells exhibited severe impact on cell viability in long-term assays. We believe that this is because EZH2 knockdown destabilizes the PRC2 complex and results in its degradation and therefore the specific impact of EZH2-miR-708 axis in metastasis cannot be accurately discerned. Therefore, in lieu of performing EZH2 knockdown, we used EZH2 histone methyl transferase inhibitor for in vivo experiments.

To determine the impact of EZH2-miR-708 axis in breast cancer metastasis to the lungs, we generated orthotopic tumors in the mammary glands of SCID mice with 1×10^6 viable MDA-LM2 cells (basal subtype, ER⁻, PR⁻, HER2⁻) (Neve et al., 2006), stably expressing luciferase and GFP transgenes. As shown in the schematic (Fig. 4), primary tumors were allowed to grow for 4 weeks (n=10/group) and then resected as described (Ryu et al., 2013). We have chosen this particular window for resection, as there are no detectable metastases in the lungs. Next, mice (10 mice/group) were treated with GSK126 (150 mg/kg, i.p. twice a week) or vehicle control (20%

captisol) for two weeks and monitored for metastases (see treatment regimen schema, **Fig. 4**). Significant reduction in metastasis was observed in drug treated group compared to controls as determined by BLI (**Fig. 4, Group 1**). This phenotype was reproduced in an independent group of mice (**Fig. 4, Group 2**). From the clinical perspective, this treatment schema mirrors an adjuvant therapy approach, with the expectation that such a treatment plan may prevent or delay the onset of distant metastases.

Milestones: stable cells with shRNA, migration, and metastasis analysis in mice (months 1-24 months):
In Progress

Local IRB/IACUC approval (Months 3):
Completed

Milestone: HRPO/ACURO Approval (Months 6):
Completed

Effect of EZH2 inhibition in breast cancer metastasis using PDX models: After having established the impact of EZH2 HMT inhibition on breast cancer metastasis in cell line-derived breast cancer models, we were interested in assessing the therapeutic benefit of EZH2 inhibitor, using a “co-clinical animal trial” design that utilizes PDX models of TNBC. Notably, these PDX models are serially propagated in mice and have maintained their triple negative marker status, molecular profiles and histological features of original human specimens. Notably, 9/21 TNBC models metastasized to the lung in concordance with patient nodal/metastatic status (Liu et al., 2010; Zhang et al., 2013). Importantly, we have determined that PDX tumors with metastatic potential in both patients and mice are associated with elevated EZH2 levels (8/8), and tumors with low metastatic potential are associated with low EZH2 (4/6). According to optimized protocols, at least one PDX model that has metastatic potential (BCM-4272, 3204, 4013, 3887, **Fig. 5A-B**) will be implanted into the mammary glands (right abdominal) of 3- to 4-week-old SCID-Beige immunocompromised mice to further studies.

PCR2 Knockdown and evaluate metastasis in vivo

1.4: Pharmacological inhibition of EZH2 (months 12-24):
In Progress (see Fig. 4)

1.5: Measure levels of miR-708, Nnat, cell migration (months 18-36):
Incomplete. We will continue these experiments in Year 2.

1.6: Chromatic IP experiments (months 18-36):

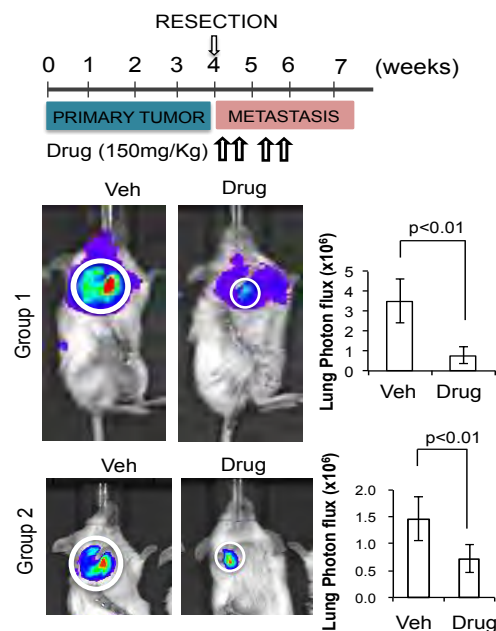


Fig. 4. EZH2 inhibition impairs metastasis. Schematic of adjuvant treatment strategy with 150 mg/kg GSK126 drug following surgical resection of primary breast tumor in mammary gland. Impact on lung metastases in two independent group of mice.

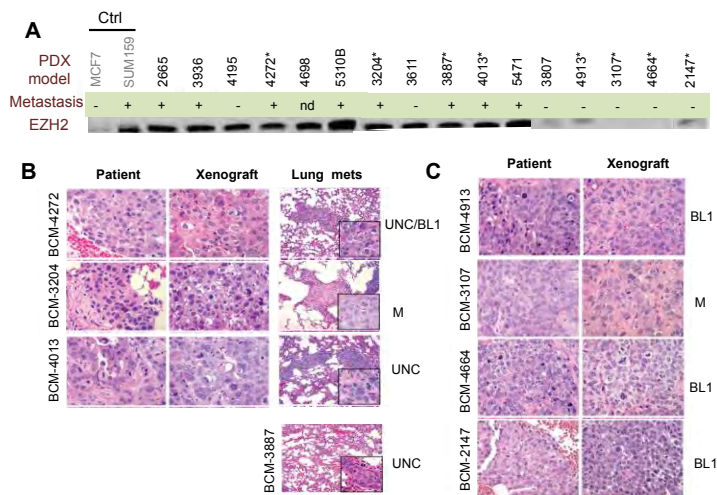


Fig. 5. Breast cancer TNBC PDX models. (A) Western blots showing EZH2 levels (normalized to actin) in a collection of PDX models. (B) Histology of EZH2^{high} PDX models that metastasize to lungs, (C), EZH2^{low} PDX models (controls). * indicates models that will be used in this study. Pietenpol classification; BL1, basal-like 1; BL2, basal-like 2; IM, immunomodulatory; M, mesenchymal-like; MSL, mesenchymal stem-like; UNC, unclassified. Panels B and C, from Zhang et al. Cancer Research 2013.

Incomplete. We will continue these experiments in Year 2.

Milestones: Show specific effects of EZH2 blockade on miR-708 and migration (months 12-36):
Incomplete

SOW AIM 2:

Assess the therapeutic potential of miR-708 against metastatic TNBC breast cancer.

2.1: MDS for delivery of miR-708 to prevent metastases derived from orthotopic tumors (months 1-12).

So far, our efforts had been more on the identification and characterization of PDX models of metastatic TNBC (see **Fig. 5**). In year 2, we will focus on the delivery of miR-708 in both the orthotopic and PDX models.

2.2: MDS for delivery of miR-708 to treat heterotransplanted patient breast cancer.

In year 2, we will focus the delivery of miR-708 in this model.

Milestone: Demonstrate that miR-708 can block TNBC mets in both models.

In year 2, we will focus on both models.

What opportunities for training and professional development has the project provided?

Opportunities for training and professional development on the project include the mentorship of post-doctoral associates to help advance their careers to faculty-level positions.

How were the results disseminated to communities of interest?

Dr. Mittal has attended breast cancer patient symposia to communicate the existence of this project and the intended goals.

What do you plan to do during the next reporting period to accomplish the goals?

In year 2, we will perform the following:

- 1) Characterize the impact of EZH2 suppression on primary breast tumors.
- 2) Chromatin IP experiments to determine EZH2 target genes.
- 3) Nanoparticle for delivery of miR-708 to prevent metastases in orthotopic models.
- 4) Nanoparticle delivery of miR-708 to prevent metastases in PDX models.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

We have established that metastatic breast cancer show increased EZH2 and decreased miR-708 levels, suggesting EZH2 epigenetically silences miR-708 in metastasis. Using both genetic and pharmacological approaches, we have demonstrated that EZH2 inhibition results in the restoration of miR-708 expression. EZH2 inhibition impacted cell migration and invasion *in vitro*, and impaired metastasis *in vivo*. These findings suggest that either inhibition of EZH2 or replacement of miR-708 have strong potential in the treatment of metastatic breast cancer including TNBC.

What was the impact on other disciplines?

This is the first study to demonstrate the impact of EZH2 inhibitor (GSK126) or miR-708 in breast cancer metastasis. This is likely to attract many investigators across disciplines in breast cancer research and result in rapid advancements towards finding a potential therapy for TNBC.

What was the impact on technology transfer?

miR-708 technology is covered in both mechanism of action and composition of matter (PCT/US2013/066376). Cornell has provided exclusive licensing to a new start up biotech company, MirCan Therapeutics, LLC, who will develop and commercialize miR-708, as a therapeutic agent for

the treatment of metastatic breast cancer including the high-risk triple negative breast cancer (TNBC).

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:

We have found that siRNA-mediated EZH2 knockdown restored expression of miR-708 (Fig. 1B), however these cells exhibited severe impact on cell viability in long term assays. We believe that this is because EZH2 knockdown destabilizes the PRC2 complex and results in its degradation and therefore the specific impact of EZH2-miR-708 axis in metastasis cannot be accurately discerned. Therefore, in lieu of performing EZH2 knockdown, we have used the EZH2 histone methyl transferase inhibitor for long term *in vitro* experiments (migration, invasion) and *in vivo* experiments (tumor growth and metastasis).

6. PRODUCTS:

Manuscript preparation is underway.

miR-708 technology is covered in both mechanism of action and composition of matter (PCT/US2013/066376). Cornell has provided exclusive licensing to a new start up biotech company, MirCan Therapeutics, LLC., who will develop and commercialize miR-708, as a therapeutic agent for the treatment of metastatic breast cancer including the high-risk triple negative breast cancer (TNBC).

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	<i>Vivek Mittal (PD) – No change</i>
Name:	<i>Linda Vahdat (Co-Investigator) – No change</i>
Name:	<i>Jenny Chang (Co-Investigator) – No change</i>
Name:	<i>Melissa Landis (Post-Doc) – No change</i>

Name:	<i>Seongho Ryu (Post-Doc) – No change</i>
Project Role:	<i>PD</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>1.8</i>
Contribution to Project:	<i>Dr. Ryu has performed all mir708 manipulations and spearheaded the experiments and all troubleshooting</i>
Funding Support:	

Name:	<i>Sharrell Lee (Technician) – No change</i>
Project Role:	<i>PD</i>
Researcher Identifier (e.g. ORCID ID):	

Nearest person month worked:	2.04
Contribution to Project:	<i>Ms. Lee has assisted in in vivo work</i>
Funding Support:	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

- 1) The project under Dr. Vivek Mittal's NCI award U54 CA143876-01 (Mittal co-PI Project1, Hempstead PI) scheduled to end on 07/31/2014 received a no cost extension until 01/31/2015. Dr. Mittal's effort is 0.12 calendar months during the NCE.
- 2) The project under Dr. Vivek Mittal's Manhasset Women's Coalition Against Breast Cancer (Mittal PI, Vahdat PI) scheduled to end on 12/31/2013 received a no cost extension until 12/31/2014. Dr. Mittal's effort is 0.24 calendar months during the NCE.
- 3) Dr. Mittal received a research award from the Mary Kay Foundation entitled, "Pharmacological targeting of the Polycomb Repressive Complex 2 impairs breast cancer metastasis" starting 07/01/2014 – 06/30/2016 on which he is spending 5% effort (0.6 calendar months).
- 4) Dr. Mittal received a one-year research award from Weill Cornell Medical College (internal seed grant) entitled, "Regulation of ERRA by the Kinesin KIF17 in Breast Epithelia" with Dr. Geri Kreitzer as the PI. Dates are from 07/01/2014 – 06/30/2015. He is spending 5% effort (0.6 calendar months).

What other organizations were involved as partners?

Organization Name: The Methodist Hospital Research Institute (TMHRI)

Location of Organization: Houston, TX

Partner's contribution to the project: Collaboration – TMHRI is a subawardee on the award.

5) SPECIAL REPORTING REQUIREMENTS

Nothing to report

6) APPENDICES:

Nothing to report